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Whole genome sequencing of respiratory syncytial (RSV) virus from clinical samples with low viral load V.1

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Stephanie Goya[1], Gabriel L. Rojo[1], Mercedes S. Nabaes Jordar[1], Laura E. Valinotto[1], Alicia S Mistchenko[1], Mariana Viegas[1]

[1]Virology Laboratory, Ricardo Gutierrez Children's Hospital, Buenos Aires, Argentina.

Stephanie Goya

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

Here is a description of a protocol for whole genome sequencing of RSV from clinical samples (nasopharyngeal aspirates -NPA-). The protocol was tested with samples with viral loads as low as 10^{+03} viral copies/ml NPA. The RNA is amplified by RT-PCR in five overlapped fragments of around 2300-4500 nt in length by using specific primers which anneal in conserved regions of the genome. Briefly the protocol includes: viral RNA extraction from NPA done with silica membrane columns and fragment amplification performed in five independent reaction tubes with OneStep RT-PCR Kit (Qiagen). Each fragment size check performed in an agarose gel electrophoresis, clean-up step done with silica membrane columns and the quantification step performed by Qubit. Finally, amplicons pooled equimolarly and library prep done with Nextera XT Kit.

Materials

MATERIALS

- Agarose **Catalog #**A5304
- DNA Clean & Concentrator™-5 **Zymo Research Catalog #**D4003
- Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851
- Ethidium bromide [EB, EtBr] **Bio Basic Inc. Catalog #**EB0195.SIZE.25g
- Agencourt Ampure XP **Beckman Coulter Catalog #**A63880
- RiboLock RNase Inhibitor **Thermo Fisher Scientific Catalog ##**EO0381
- Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096
- OneStep RT-PCR Kit **Qiagen Catalog #**210210
- PureLink viral RNA/DNA mini kit **Invitrogen - Thermo Fisher Catalog #**12280050
- QIAamp Viral RNA Mini Kit **Qiagen Catalog #**52904

Safety warnings

Ethidium bromide is a potent mutagen. Ethidium bromide solution must be handled with extreme caution and decontaminated prior to disposal.

Viral RNA extraction

1 Centrifuge the nasopharyngeal aspirate (NPA) at 5,000 g for 5 min to remove cellular debris.

2 Extract viral RNA from 200 µl of NPA by following the protocol PureLink viral RNA/DNA mini kit (Thermo Fisher Scientific) according the manufacturer's instructions except for 2.8 µl of carrier RNA instead of 5.6 µl as recommended to reduce the presence of tRNA in the extracted RNA. Nevertheless, 140 µl of NPA should be used if QIAamp Viral RNA Mini Kit (Qiagen) is used instead of PureLink kit. In this case, no changes from the protocol should be done.

3 Elute viral-extracted RNA in 40 µl of DNase/RNase-free water. Add 1.25 µl of 40 U/µL RiboLock RNase Inhibitor (Thermo Fisher Scientific) to preserve the extracted RNA.

4 Extracted RNA can be storage at -80ºC.

RT-PCR

5 **PRIMERS:**
Fragment 1: 2384 bp
Forward 1f: 5'-ACGCGAAAAAATGCGTACwAC-3'
Reverse 2384r: 5'-GCrTCTTCTCCATGrAATTC-3'
Fragment 2: 2731 bp
Forward 2124f: 5'- GCwGGyCTAGGCATAATG-3'
Reverse 4875r: 5'- GTTGTTrGTGTrACTTTGT-3'
Fragment 3: 4351 bp
Forward 3314f: 5'-AyCCyGCATCACTwACAAT-3'
Reverse 7665r: 5'-CAGGAAACAGCTATGACCyAAGCA-3'
Fragment 4: 4114 bp
Forward 7094f: 5'-TGATGCATCAATATCTCAAGTC-3'
Reverse 11208r: 5'-CTCCTGTGTTAAGCTACCTATAG-3'
Fragment 5: 4331 bp
Forward 10438f: 5'-AGTyTkACAAGATATGGTGATCT-3'
Reverse 15180r: 5'-AAGTGTCAAAAACTAATATCTCGT-3'

All the procedure should be done in ice.
Amplify each fragment in an independent RT-PCR reaction with OneStep RT-PCR kit of Qiagen by mixing:
primer f (25 µM)--- 0.6 µl

primer r (25 µM)--- 0.6 µl
RNA--- 6 µl

Incubate at 65 °C for 5 min, then place 2 min in ice.

Add (master mix can be done):
H2O DNase/RNase free--- 5 µl
Buffer 5X--- 10.55 µl
RNAsa Inhibitor (40 U/µl)---0.25 µl
dNTP mix (10 mM)--- 1 µl
Enzime Mix--- 1 µl
(Final volume: 25 µl)

Incubate:
45 °C 30 min
95 °C 15 min
40 times: 94 °C 10 sec
55 °C 30 sec
68 °C 4.5 min
68 °C 10 min
4 °C hold

Fragment verification, quantification and pool

6 Run 4 µl of RT-PCR product on a 1.8% agarose gel stained with Ethidium Bromide.

7 Perform a clean-up of RT-PCR product with DNA Clean & Concentrator kit (Zymo Research) following the manufacturer's instructions. Elute with water PCR-grade, avoid elution with buffer containing EDTA.

8 Run 2 µl of each amplicon in a Qubit dsDNA HS Assay.

9 Pool equimolarly the 5 amplicons

NGS

10 Follow NexteraXT kit (Illumina) to perform library preparation.
After index addition by amplification and clean-up with Agencourt Ampure XP, quantify the libraries with the Qubit dsDNA HS Assay Kit.

Perform a standard normalization instead of beads normalization. Never normalize with beads when libraries have a concentration lower than 15 nM
If you are not familiar with bioinformatic analysis in NGS, we recommend using UGENE or Geneious.